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| (54) Title: GLYCOSAMINOGLYCAN DERIVATIVES AND THEIR USE AS INHIBITORS OF TUMOR INVASIVENESS OR METASTATIC PROFUSION   |  |   |   |
| (57) Abstract<br><p>Method for impeding the formation of tumor metastasis or tumor invasiveness in a host. Such inhibition comprises administration to the host of a glycosaminoglycan derivative substantially devoid of anticoagulation activity and is an effective inhibitory of heparanase activity. Such a glycosaminoglycan derivative may be provided by purchase or synthesis as directed herein. Parenteral administration to a tumor-bearing host of the glycosaminoglycan derivative results in the exposure of host-borne tumor cells thereto. Such exposure to effective levels of the derivative results in the inhibition of tumor heparanase activity and a lessening of invasiveness and metastatic spread. Heparin, a glycosaminoglycan particularly effective as a heparanase inhibitor and an anti-clotting agent, is a preferred glycosaminoglycan for derivatization. Upon derivatization according to the present invention heparin may be converted into a glycosaminoglycan derivative substantially devoid of anticoagulant activity but yet being an effective inhibitor of heparanase activity. Mere reduction of heparin carboxyl groups results in the production of a glycosaminoglycan derivative inhibitory to heparanase activity but without substantially anticoagulant activity non-anticoagulating, heparanase-inhibiting glycosaminoglycan derivatives may also be prepared from heparin, for example, by: at least partial N-desulfation and then N-acetylation; or N-, O-desulfation followed by N-resulfation.</p> |  |   |   |

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GLYCOSAMINOGLYCAN DERIVATIVES AND THEIR  
USE AS INHIBITORS OF TUMOR INVASIVENESS  
OR METASTATIC PROFUSION

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The present invention relates to glycosaminoglycan derivatives useful in the inhibition of endoglycosidase activity and of tumor invasiveness or metastatic spread.

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A class of biological substances called the proteoglycans form the ground substance in the extracellular matrix of connective tissues. These proteoglycans are polyanionic substances of high molecular weight and contain many different types of heteropolysaccharide side chains covalently linked to a polypeptide backbone. These proteoglycans may contain over 95% carbohydrates. The polysaccharide groups of the proteoglycans were formerly called mucopolysaccharides but now are preferably termed

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glycosaminoglycans since all contain derivatives of glucosamine or galactosamine.

5 A variety of enzymes may be involved in the normal metabolic degradation of proteoglycans. Initial proteoglycan degradation often involves proteolysis to separate or digest protein components. Such proteolysis results in the production of glycosaminoglycans. The glycosaminoglycans in turn are subject to glycosaminoglycan endoglycosidase enzymic action which produces smaller glycosaminoglycan fragments. The glycosaminoglycans or fragments thereof are subject to glycosaminoglycan exoglycosidase enzymic action which produces monosaccharides from the non-reducing ends of glycosaminoglycans.

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An increasing interest in the endoglycosidases has arisen in recent years because of a possible relationship of these enzymes with tumor invasiveness and tumor metastatic activity. Nicolson (1982, Biochem. Biophys. Acta. V 695, pp 113-176) reviewed a variety of oligosaccharide-degrading enzymes (pp 141-142) reported to be of interest in malignant disease. Nicolson (1982, J. Histochem. & Cytochem. V 30, pp 214-220) described a proposed mechanism for tumor cell invasion of endothelial cell basal lamina and a related production of degradation products from proteins and glycosaminoglycans. Kramer et al., (1982, J. Biol. Chem. V 257, pp 2678-2686) reported a tumor-derived glycosidase capable of cleaving specifically glycosaminoglycans and releasing heparan sulfate-rich fragments.

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Irimura et al., (1983a, Analyt. Biochem. V 130, pp 461-468) describe high-speed gel-permeation chromatography of glycosaminoglycans. Heparan sulfate degrading activity of melanoma cells was measured by using this chromatographic procedure. Nakajima et al., (1983, Science, V 220, pp 611-613) described a relationship of metastatic

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activity and heparan sulfate degrading activity in melanoma cell lines. The disappearance of higher molecular weight heparan sulfate was followed by polyacrylamide gel electrophoresis, staining and densitometry.

5 Vlodavsky et al., (1983, Cancer Res. V 43, pp 2704-2711) described the degradation by two T-lymphoma cell lines of  $^{35}\text{S}$  labeled proteoglycans from confluent endothelial cells. The highly metastatic line had much higher  
10  $^{35}\text{S}$  liberating activity than did the low metastatic line.

Irimura et al., (1983c, Proc. Am. Soc. Cancer Res. V 24, p 37, abstract 144), using high performance liquid chromatography, describe heparan sulfate degradative  
15 enzyme activity of melanoma cells. Nakajima et al., (1984, J. Biol. Chem. V 259, pp 2283-2290) describe characterizations of metastatic melanoma heparanase. High speed gel permeation chromatography and chemical analyses were used in a description of functional substrates and  
20 products formed. Nakajima et al. (1986, Anal. Biochem., in press) synthesized a solid-phase substrate for heparanase by crosslinking radiolabeled and reductively aminated HS to amino-reactive agarose beads via one covalent linkage. This solid-phase substrate was used for  
25 the measurement of heparanase activity in various human melanoma cell lines (Nakajima et al., (1986) Cancer Letters, V 31, pp 277-283) and sera from mammary adenocarcinoma-bearing rats and malignant melanoma patients (Nakajima et al., (1986) In: Cancer Metastasis :  
30 Experimental and Clinical Strategies, D.R. Welch, B.K. Bhuyan, L.A. Liotta, eds. Alan R. Liss, Inc., New York, pp 113-122).

From the foregoing it may be seen that significant  
35 interest exists in convenient, accurate and reproducible endoglycosidase assays and production of potent heparanase

inhibitors, particularly since endoglycosidases may play critical roles in the establishment of tumor metastases.

The ability of tumor cells to invade host tissues and metastasize to distant, often specific organ sites, is one of their most important properties. Metastasis formation occurs via a complex series of unique interactions between tumor cells and normal host tissues and cells. These processes involve several discrete and selective steps such as: invasion of surrounding tissues, penetration of lymphatics of blood vessels and transport in lymph or blood, or dissemination into a serous cavity, arrest and invasion at distant sites, and survival and growth to form secondary lesions.

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Basement membranes are continuous sheets of extracellular matrix composed of collagenous and non-collagenous proteins and proteoglycans that separate parenchymal cells from underlying interstitial connective tissue. They have characteristic permeabilities and play a role in maintaining tissue architecture. Metastasizing tumor cells must penetrate epithelial and endothelial basement membranes during invasion and metastasis, and the penetration and destruction of basement membranes by invasive tumor cells has been observed using electron microscopy. Since basement membranes are rigid structures formed from unique sets of macromolecules, including type IV collagen, laminin, heparan sulfate (HS), proteoglycan and fibronectin, the successful penetration of a basement membrane barrier probably requires the active participation of more than one tumor cell-associated enzyme.

Due to its unique physical and chemical properties such as its polyanionic character and barrier properties against macromolecules (Kanwar et al., 1980 J. Cell. Biol. V 86, pp 688-693), HS is an important structural component

of basement membranes. HS binds to fibronectin, laminin and type IV collagen, and these molecules have been collectively observed in the basal lamina using antibodies raised against each component. HS may be involved in  
5 basal lamina matrix assembly by promoting the interactions of collagenous and non-collagenous protein components while protecting them against proteolytic attack. Thus, the destruction of HS proteoglycan barrier could be important in basement membrane invasion by tumor cells.

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The interactions between malignant cells and vascular endothelium have been studied using monolayers of cultured vascular endothelial cells that synthesize an extra-cellular matrix resembling a basement membrane. With this  
15 model, it has been found that metastatic B16 melanoma cells degrade matrix glycoproteins, such as fibronectin, and matrix sulfated glycosaminoglycans, such as heparan sulfate. Since HS was released in solution as fragments approximately one-third their original size, it has been  
20 proposed that metastatic tumor cells characteristically have a HS endoglycosidase.

The relation between metastatic properties and the ability of five B16 melanoma sublines of various implanta-  
25 tion and invasion characteristics to enzymatically degrade subendothelial extracellular matrix indicated that highly invasive and metastatic B16 sublines degraded sulfated glycosaminoglycans faster than did sublines of lower metastatic potential (Nakajima et al., (1983), Science V  
30 220, p 611), and intact B16 cells (or their cell-free homogenates) with a high potential for lung colonization also degraded purified heparan sulfate at higher rates than did B16 cells with a poor potential for lung coloni-  
zation (ibid).

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The abilities of B16 cells to degrade HS from various origins and other purified glycosaminoglycans (heparin, chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, keratan sulfate, and hyaluronic acid) have been studied. In order to analyze glycosaminoglycan degradation products, an analytic procedure was developed using high-speed gel permeation chromatography (Irimura et al., (1983a) Anal. Biochem. V 130, p 161; Nakajima et al., (1984) J. Biol. Chem. V 259, p 2283). HS metabolically labeled with  $^{35}\text{S}$ -sulfate was purified from basement membrane producing EHS sarcoma and PYS-2 carcinoma cells, and subendothelial matrices of bovine aortic endothelial (BAE) and corneal endothelial (BCE) cells (ibid). HS molecules purified from bovine lung and other glycosaminoglycans were labeled with tritium at their reducing termini using  $^3\text{H}$ - $\text{NaBH}_4$ . These labeled glycosaminoglycans were incubated with B16 cell extracts in the absence or presence of D-saccharic acid 1,4-lactone, a potent exo-beta-glucuronidase inhibitor, and degradation fragments were analyzed by high-speed gel permeation chromatography.

HS isolated from the various origins described above were all degraded into fragments of characteristic molecular weight, in contrast to hyaluronic acid, chondroitin 6-sulfate, chondroitin 4-sulfate, dermatan sulfate, keratan sulfate, and heparin, which were essentially undegraded. Heparin, but not other glycosaminoglycans, inhibited HS degradation. The time dependence of HS degradation into particular molecular weight fragments indicated that melanoma heparanase cleaves HS at specific intrachain sites (ibid). In order to determine specific HS cleavage points, the newly formed reducing termini of HS fragments were investigated by: labeling with  $^3\text{H}$ - $\text{NaBH}_4$ ; hydrolysis to monosaccharides; and analysis of these saccharides by paper chromatography. Since  $^3\text{H}$ -reduced terminal monosaccharides from HS fragments were



overwhelmingly (>90%) L-gulonic acid, the HS-degrading enzyme responsible was an endoglucuronidase (heparanase).

HS-degrading endoglucuronidases have been found in various tissues, such as human skin fibroblasts, rat liver cells, human placenta, and human platelets. HS-degrading endoglucuronidases in mammalian cells were reported previously by other investigators to be "heparitinases" to indicate heparitin sulfate (heparan sulfate)-specific endoglycosidase. However, heparitinase originally was used to designate an elimination enzyme (EC 4.2.2.8) in Flavobacterium heparinum, and this enzyme cleaves non-sulfate and monosulfated 2-acetoamido-2-deoxy-alpha-D-glucosyl-D-hexuronic acid linkages of HS. Since HS-specific endoglycosidases in mammalian cells are endoglucuronidases, except for one found in skin fibroblasts, it was proposed that mammalian cell endoglucuronidases capable of degrading HS should be called "heparanases", consistent with the currently used term "heparan sulfate".

20

High heparanase activity in human melanoma cells was demonstrated using a solid-phase substrate, partially N-desulfated N-[<sup>14</sup>C] acetylated HS crosslinked to agarose beads via one covalent linkage (Nakajima et al., (1986) Cancer Letters, V 31, pp 277-283; Nakajima et al., (1986) Anal. Biochem. in press). All of the 15 human melanoma cell lines tested were found to have heparanase activity and almost all possessed high activities comparable or greater than that of the murine B16-F1 melanoma line. Human A375 melanoma variants of high lung metastatic potential in athymic nude mice had significantly higher heparanase activities than did A375 parental cells of low metastatic potential.

High heparanase activity was also found in the sera from highly metastatic tumor-bearing animals and malignant

melanoma patients (Nakajima et al, (1986) In: Cancer Metastasis : Experimental and Clinical Strategies, D.R. Welch, B.K. Bhuyan, L.A. Liotta, eds., Alan R. Liss, Inc., New York, pp 113-122). A significant difference in serum heparanase activity was found between a group of normal adults and a group of malignant melanoma patients (N=35,  $p < 0.05$ ). The mean values of serum heparanase activities in normal adults and malignant melanoma patients were 3.97 and 9.43 mg HS/h/ml serum, respectively. Some of the patients having documented lymph node metastases had 4 to 6-fold higher serum heparanase activities than normal adults.

Anticoagulants, such as heparin, warfarin, dextran sulfate and Ancrod; (Agkistrodon rhodostoma venom protease, Abbott) have been used to prevent blood coagulation and reduce the formation of metastatic tumor cell thrombi in the blood which lodge more effectively in the microcirculation Hilgard et al., Eur. J. Cancer, V 12, pp 755-762, 1976). H. Ludwig (Gynakologe, V 7, pp 1-10, 1974) reported longer recurrence-free intervals and less metastases in patients during irradiation of their gynaecological cancers. Heparin has been used as an adjuvant therapy agent with combination chemotherapy of inoperable lung cancer with enhanced therapeutic effects (Elias, Proc. Amer. Assoc. Cancer Res., V 14, p 26, 1973, Abst.). L. Michaels (Lancet, V 2, pp 832-83, 1964) found that the cancer incidence and death rate was lower than expected for patients receiving heparin. Dextran sulfate ( $M_r - 7,000$ ) has been used to inhibit metastasis of rat lung tumors, and human lung cancer patients have received long term oral dextran sulfate. Although the anti-metastatic effects of dextran sulfate were marginal in cancer patients, this was probably due to the low intestinal absorption of dextran sulfate (Suemasu, Gann Monogr., V 20. pp 163-172, 1977).

Heparin and related sulfated glycoconjugates with anticoagulation properties, such as dextran sulfate have been used experimentally as antimetastatic agents (Tsubura et al., (1977) Gann Monogr. Cancer Res., V 20, pp 147-153; 5 Hilgard, (1984) in Cancer Invasion and Metastasis, Biologic and Therapeutic Aspects, Nicolson et al., eds, pp 353-360 Raven Press, N.Y.) The basis for this use was the assumption that platelet aggregation, together with activation of the coagulation cascade, enhanced the formation of 10 tumor embolism and increased implantation and metastatic colonization of blood-borne tumor cells. In other studies on the effects of heparin on metastasis, however, heparin administration increased, decreased, or had no effect on tumor cell dissemination and organ colonization, depending 15 on the experimental system. Mechanisms other than the anticoagulation effects of heparin on tumor metastasis were suggested by these results, but the possible involvement of tumor heparanase had not been considered. The present invention relates to heparin derivatives without 20 anticoagulant properties and which inhibit the heparanase activity of metastatic mouse melanoma cells. These substances were useful as tools for in vitro and in vivo studies involving the role of heparanase in tumor invasion and metastasis.

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Using oral administration of heparin in combination with hydrocortisone, it was reported that complete regression of established transplantable tumors in mice could occur through inhibition of tumor angiogenesis 30 (Folkman et al., Science, V 221, pp 719-725, 1983). This suggested that anti-angiogenic substances could be used for cancer therapy (J. Folkman in: Important Advances in Oncology, de Vita et al., eds, pp 42-62, J.B. Lippincott, Philadelphia, 1985). In such studies heparin was 35 administered in the drinking water of animals. For example, hamsters have been inoculated with transplantable

pancreatic carcinoma cells and have been treated by receiving heparin or hexuronyl hexaminoglycan sulfate, a heparin derivative, in their drinking water at concentrations of 10 mg/ml with hydrocortisone (0.5 mg/ml). After  
5 treatment for 6-9 days, the tumors were examined. In 3 out of 4 of the tumors the hexuronyl hexaminoglycan sulfate plus hydrocortisone resulted in significant reductions in growth rate in vivo and significant inhibitions of capillary endothelial cell migration in  
10 Boyden chambers in vitro (Rong et al., Cancer, V 57, pp 586-590, 1986).

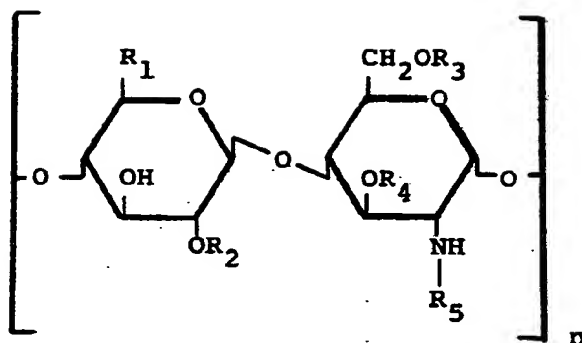
Proliferation of vascular smooth muscle cells has been shown to be an important step in the pathogenesis of  
15 arteriosclerosis (Ross et al., Science, V 180, pp 1332-1339, 1973). Commercial heparin preparations have been separated into anticoagulant and non-anticoagulant mixtures by use of antithrombin to remove the anti-coagulant heparin. Both of these forms of heparin signi-  
20 ficantly inhibit the growth of smooth muscle cells in vitro (Hoover et al., Circulation Res., V 47, pp 578-583, 1983). Using the heparin preparations at a concentration of 10 ug/ml resulted in approximately 50% inhibition of <sup>3</sup>H-thymidine uptake by arterial smooth muscle cells, which  
25 is indicative of growth inhibition. Administration of anticoagulating and non-anticoagulating heparin fractions inhibited intimal smooth muscle proliferation, as determined by the total plaque volume two weeks after arterial injury. Non-anticoagulating heparin given at a dose of  
30 100 USP units per kg body weight per hour in Sprague-Dawley rats resulted in 77% inhibition of myointimal growth (Guyton et al., Circulation Res., V 46, pp 625-634, 1980).

35 Further studies indicated that the minimum fragment size of hepain, which was growth inhibitory toward

vascular smooth muscle cells, in a hexasaccharide, and the maximum anti-proliferative activity was obtained with a 12 residue heparin. Most modified heparins (totally desulfated, N-desulfated, and totally desulfated re-N-  
5 acetylated) lost their anti-proliferative activity, but the N-desulfated N-resulfated heparin and the N-desulfated N-acetylated heparin retained full growth inhibitory properties (Castellot et al., J. Cell. Physiol., V 120, pp 315-320, 1984). Diseases, such as arteriosclerosis,  
10 develop over a long period of time. Therefore, the main use of such treatments might be in vascular damage due to trauma or surgery, such as artery vein grafts or arterio-venous shunts for kidney dialysis.

15       The present invention comprises novel compositions and a method for impeding the formation of tumor metastasis or tumor invasiveness in a host. The method comprises parenteral administration to the host of a glycosaminoglycan derivative substantially devoid of anticoagulation  
20 activity and which is an effective inhibitor of heparanase activity. Suitable glycosaminoglycan derivatives, which are useful as novel compositions in the method of the invention, include glycosaminoglycan derivatives of the following structure:

Figure 1



Where

$R_1$  is  $-\text{COOH}$  or  $-\text{CH}_2\text{OH}$  and the configuration of the carbon atom to which  $R_1$  is bound is  $\underline{D}$  or  $\underline{L}$ ;

$R_2$  is  $-\text{H}$  or  $-\text{SO}_3^-$

$R_3$  is  $-\text{H}$  or  $-\text{SO}_3^-$ ;

$R_4$  is  $-\text{H}$  or  $-\text{SO}_3^-$ ;

$R_5$  is  $-\text{H}$ ,  $-\text{SO}_3^-$  or  $-\text{CO}-\text{CH}_3$ ;

$n$  is 3 to 30; and

each of the terminal monomeric units

is a monomeric repeating unit with the terminal oxygen atom being bound to a blocking group.

The blocking group may be a small alkyl or acyl group having less than about 5 carbon atoms and preferably is -H, -CH<sub>3</sub>, -SO<sub>3</sub><sup>-</sup> or CO-CH<sub>3</sub>.

5        The glycosaminoglycan derivatives of the present invention may be prepared by derivatization of heparin obtained from natural sources or they may be prepared by a variety of conventional synthetic techniques. Administration to a tumor-bearing host of an effective  
10 amount of a glycosaminoglycan derivative of the invention results in the exposure of host-borne tumor cells thereto. Such exposure to effective levels of the derivative results in the inhibition of tumor heparanase activity and a lessening of tumor invasiveness and metastatic spread.

15        Heparin, a glycosaminoglycan which is effective both as a heparanase inhibitor and an anti-clotting agent, is a preferred glycosaminoglycan for derivatization. Upon derivatization according to the present invention,  
20 heparin may be converted into a glycosaminoglycan derivative substantially devoid of anticoagulant activity but yet being an effective inhibitor of heparanase activity. Reduction of heparin carboxyl groups results in the production of a glycosaminoglycan derivative inhibitory to  
25 heparanase activity but without substantially anticoagulant activity. Heparanase-inhibiting glycosaminoglycan derivatives having no substantial anti-coagulant activity may also be prepared from heparin, for example, by: at least partial N-desulfation and then N-acetylation; or at  
30 least partial N-, O-desulfation followed by N-resulfation.

FIGURE 1: Monomeric structural formulas which  
35 represent derivatives of heparin used in this study (actual structures obtained by derivatization are averages

of monomeric units shown). a, Intact heparin; b, Partially N-desulfated heparin; c, completely N-desulfated heparin; d, N-acetylated N-desulfated heparin; e, N- and O-desulfated heparin; f, N-acetylated N- and O-desulfated heparin; g, N-resulfated N- and O-desulfated heparin; h, carboxy-reduced heparin.

FIGURE 2: Cellulose acetate sheet electrophoresis of chemically modified heparins. The sheet is 7.6 cm in total length, with 1.5 cm of stacking area blotted with water and 5.0 cm of separating area blotted with a 0.5M pyridine/0.5M acetic acid, pH 5.0 buffer. The electrodes are in the same buffer. Electrophoresis is performed under constant voltage (12 V/cm) at 4°C for 45 min. After electrophoresis, the sheet was stained with toluidine blue (0.1% toluidine blue in 1% acetic acid) and destained with 1% acetic acid.

FIGURE 3: HPLC of chemically modified heparin on a single 2.7 x 75 cm column of Fractogel TSK HW-55(S) (Irimura et al., 1983). Elution was accomplished with 0.2M sodium chloride at a flow rate of 0.75 ml/min. During the separation the column was kept at 55°C by a water jacket. Each sample was dissolved in water (5 ug/ml) and 25 ul was injected. Patterns a-h indicate the analytical results of derivatized heparins shown in Figure 1.

FIGURE 4: Results of HPLC assay of B16 melanoma heparanase activity in the presence of chemically modified heparin derivatives. A high-speed gel-permeation chromatography system was equipped with a 0.70 x 75 cm column of Fractogel TSK-HW55(S). Elution was performed with 0.2 M NaCl at a flow rate of 1.0 ml/min at 55°C. Each 0.5-ml fraction corresponding to 30-sec elution was collected, mixed with 3 ml of Hydrofluor and its radioactivity



determined. Panel A,  $^{14}\text{C}$ -HS was incubated with heat-inactivated crude heparanase ( $10^6$  B16-BL6-melanoma cell equivalents) at  $37^\circ\text{C}$  for 6 h, and analyzed. B, same incubation as A except that the enzyme was not heat-inactivated. C, same incubation and analysis as B, but 1 mg/ml of heparin was added to the incubation mixture. D, same incubation and analysis as B, but with 1 mg/ml of completely N-desulfated heparin added to the incubation mixture. E, same incubation and analysis as B, with 1 mg/ml N- and O-desulfated heparin added to the mixture. F, same as B, but with 1 mg/ml of N-resulfated N- and O-desulfated heparin added.

FIGURE 5: Inhibitory activities of chemically modified heparins on release of  $^3\text{H}$ -acetylated HS fragments by metastatic B16 melanoma heparanase. The measurements were performed by incubating B16-BL6 melanoma heparanase. The measurements were performed by incubating B16-BL6 melanoma cell lysates equivalent to  $10^6$  cells with a suspension of  $^3\text{H}$ -HS-agarose beads prepared by partial N-desulfation and  $^3\text{H}$ -acetylation (approximately 20% suspension), and various concentrations of heparanase inhibitors at  $37^\circ\text{C}$  for 24 hr with gentle mixing. Panels a-h indicate the results using the 8 different chemically modified heparins as indicated in Figures 1 and 2. The inhibitory activities are indicated by the percent release of HS fragments compared to the release without added inhibitors.

FIGURE 6: Effects of chemically modified heparins on the release of  $^{35}\text{S}$  labeled substances from the extracellular matrix of mouse lung capillary endothelial cells in vitro. Heparin and some chemically modified heparins induced non-enzymatic release of  $^{35}\text{S}$  labeled materials in the absence of B16 melanoma cells, while they inhibited the degradation of these macro molecules caused

by B16 melanoma cells. (See: Nakajima et al., Science 220:611-613, 1983, for the methods.)

FIGURE 7: Effects of chemically modified heparins on the incorporation of  $^3\text{H}$ -thymidine to B16-BL6 melanoma cells grown in tissue culture. (See: Irimura et al., (1981) Cancer Res. 41:3411-3418, for the methods.)

FIGURE 8: Adhesion of B16 melanoma cells to bovine aortic endothelial cell monolayers. (a) B16 melanoma cells were pretreated with modified heparins at room temperature for 2 hrs, and the adhesion assays were performed in the conteneous presence of these compounds at 37°C. (b) Endothelial cells were pretreated with modified heparins at 37°C for 2 hrs, then adhesion assays were performed. (See Nicolson and Custead, (1985) Cancer Res. 45:331-336, for the methods)

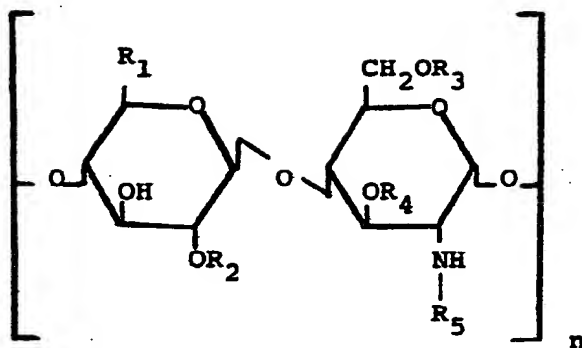
FIGURE 9: Effects of modified heparins on the adhesion of B16-F10 melanoma cells to the syngenic mouse lung tissues in vitro. Primary organ cultures of mincd mouse lung tissues were mixed with melanoma cell suspensions and incubaed at 37°C under gentle gyration. The attached melanoma cells to the lung tissues were histologically quantitated after sectioning. (See Nicolson et al., (1985) Invasion Metastasis, 5:144-158, for detailed methods.)

FIGURE 10: Effects of modified heparins on the B16-F10 melanoma cell-invasion of isolated mouse lung tissues in vitro. The experimental conditions were the same as the adhesion experiments. The invaded cells were counted on 4 um sections under a light microscope. (See Nicolson et al., (1985) Invasion Metastasis, 5:144-158, for detailed methods.)

Heparanase from metastatic melanoma cells is an endo-beta-glucuronidase which is specific for HS (Irimura et al., (1983b), Gann Monogr. Cancer Res., V 29, pp 35-46; Nakajima et al., (1984) J. Biol. Chem, V 259, pp 2283-  
5 2290). Although heparin is structurally and biosynthetically related to HS, it is a poor substrate for heparanase, and it interferes with HS degradation (Nakajima et al., (1984)). Structural differences between heparin and HS are based primarily on the degrees of  
10 sulfation of glucosamine residues, and the relative contents of iduronic acid. The heparanase-inhibitory activity of heparin should be determined, therefore, by its sulfate as well as its carboxy groups. If one of these group were responsible for heparanase inhibition,  
15 this information should be useful in developing specific heparanase inhibitors. Furthermore, since sulfamine and O-sulfate groups of glucosamine, O-sulfate groups of iduronic acid, and carboxy groups of uronic acid are essential for heparin's anticoagulation activities, some  
20 of the chemical modifications should produce heparin derivatives that inhibit heparanase activity but are not anticoagulants.

The glycosaminoglycan derivatives of the invention  
25 are derivatives of heparin or heparin analogs which are effective inhibitors of heparanase while being substantially devoid of the anticoagulant activity that is characteristic of heparin. In particular, preferred compounds of the invention are glycosaminoglycan  
30 derivatives of the following structure:

FIGURE 2



Where

$R_1$  is  $-\text{COOH}$  or  $-\text{CH}_2\text{OH}$  and the

configuration of the carbon atom to which  $R_1$  is bound is  $D$  or  $L$ ;

$R_2$  is  $-\text{H}$  or  $-\text{SO}_3^-$ ;

$R_3$  is  $-\text{H}$  or  $-\text{SO}_3^-$ ;

$R_4$  is  $-\text{H}$  or  $-\text{SO}_3^-$ ;

$R_5$  is  $-\text{SO}_3^-$  or  $-\text{CO}-\text{CH}_3$ ; and

$n = 3-30$ ;

provided that: when  $R_1$  is  $-\text{CH}_2\text{OH}$ , at least 50% of  $R_2$ ,  $R_3$  and  $R_5$  is  $-\text{SO}_3^-$ ;

when  $R_1$  is  $-\text{COOH}$ , and  $R_2$ ,  $R_3$  and  $R_4$  are  $-\text{H}$ ,  $R_5$  is  $-\text{SO}_3^-$ ;

when  $R_1$  is  $-\text{COOH}$ , and at least 50% of  $R_2$ ,  $R_3$  and

$R_4$  is  $-\text{SO}_3^-$ ,  $R_5$  is  $-\text{CO}-\text{CH}_3$ ; and

each of the terminal monomeric units is a repeating monomeric unit having a terminal oxygen atom bound to a blocking group.

substantially devoid of anticoagulant activity. A generally desirable chemical property of usable cancer-inhibiting glycosaminoglycan substances is the presence of sulfated amino groups.

5

In a general sense the cancer-inhibiting substances of the present invention may be synthesized by, for example:

10       (1) First, identifying a material, usually a sulfated glycosaminoglycan, which inhibits heparanase and has anticoagulation activity. Then, chemically altering this material to substantially remove anticoagulation activity while enhancing or at least not removing  
15 heparanase-inhibitory activity. Many of the Examples appended hereto illustrate this approach; or,

          (2) First, obtaining a glycosaminoglycan which substantially neither has anticoagulant activity nor  
20 inhibits heparanase. Then, chemically altering the glycosaminoglycan, for example by sulfation of amino groups, to produce a glycosaminoglycan derivative having heparanase inhibitory activity but being substantially devoid of anticoagulant activity.

25

Chemically modified substances may be assayed by methods described herein to follow effects upon anticoagulant activity and heparanase-inhibitory activity. When derivatives having these properties are non-toxic at  
30 levels adequate to inhibit heparanase activity are obtained, they would be suitable for the practice of the methods of the present invention.

In chemotherapeutic usage, the cancer-inhibiting  
35 substances of the present invention will be administered parenterally at dosages between about 30 mg/day and about

250 mg/day, preferably between about 30 mg/day and about 100 mg/day. However, some variation in dosage will necessarily occur depending on the condition of the subject being treated and the potency of the particular substance. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

In breast cancer, adjuvant chemotherapy, with or without hormone treatment, has been used for treatment of operable breast cancer (Fisher et al., NCI Monograph, V 1, pp 35-43, 1986). At M.D. Anderson Hospital, adjuvant chemotherapy trials have utilized, as a minimum treatment protocol, a program consisting of 5-fluorouracil, doxorubicin, and cyclophosphamid (Buzdar et al., NCI Monograph, V 1, pp 81-85, 1986). In more recent studies cycles of chemotherapy were repeated at 28 day intervals using continuous infusion pumps to administer the chemotherapeutic agents. In breast cancer, administration of non-anticoagulation heparin derivatives may be used at two times during therapy: (1) during surgery of the primary tumor and post-operatively to inhibit the dissemination of cancer cells caused by surgical manipulation, and (2) during the continuous infusion of chemotherapeutic drugs, to prevent tumor cell dissemination in the blood and capillary lodgement caused by drug damage of normal tissues, such as endothelial cells (Nicolson et al., Cancer Res., V 45, pp 331-336, 1985). The heparin derivatives would be administered intravenously in amounts between about 30 mg/day and about 250 mg/day, preferably between 30 mg/day and about 100 mg/day. However, some variation in dosage will necessarily occur depending upon the condition of the subject being treated and the potency of the particular substance. The physician responsible for administration will determine the appropriate dose for the individual subject.

In malignant melanoma the following example of adjuvant therapy has been used to treat metastatic disease. Patients have been treated with an adjuvant sequence of cisplatin, vinblastine and bleomycin intravenously every three weeks for a total of three cycles (Johnson et al., Cancer Treatment Rep., V 69, pp 821-824, 1985). The heparin derivatives would be administered intravenously, as described above, during administration of the chemotherapeutic agents.

10

The novel compounds of the invention may be administered alone or in combination with pharmaceutically acceptable carriers and will typically be formulated in a unit injectable dosage form (solution, suspension, emulsion), preferably in a pharmaceutically acceptable carrier which is inherently non-toxic and non-antigenic. In this regard, suitable carriers for formulations in accordance with the invention include saline, Ringer's solution, mannitol, dextrose solution, and normal serum albumin. Non-aqueous carriers such as fixed oils and ethyl oleate may also be used. The carrier may also contain small amounts of additives such as substances which enhance isotonicity and chemical stability, for example, buffers and preservatives. In any case, the formulations in accordance with the invention shall contain an effective amount of glycosaminoglycan derivative to impede the spread of cancer cells by metastases or invasion.

30        These aqueous solutions are especially suitable for parenteral administration. In this connection, the sterile aqueous media employed are all readily available by standard techniques known to those skilled in the art. Treatment in accordance with the invention is performed in a manner such that a maximal inhibition of tumor spread will occur. This will involve the intraperitoneal, intra-

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venous, intraarterial or intramuscular injection of the maximum tolerated dose of glycosaminoglycan derivative which is effective in invoking the desired inhibition of cancer cell spread in the patient.

5

The following examples are presented to describe preferred embodiments and utilities of the present invention and are not meant to limit the present invention unless otherwise stated in the claims appended hereto.

10

#### EXAMPLE 1

#### MATERIALS AND METHODS

15

Chemical Modification of Heparin. Chemical modifications and the resultant compounds used in this study are schematically shown in Figure 1. Heparin from porcine intestinal mucosa (sodium salt) was purchased from Sigma Chemical Co. (St. Louis, MO.). One gram of heparin was dissolved in 30 ml of water and applied to a 1.5 x 8 cm column of Dowex 50W X 8, 50-100 mesh ( $H^+$  form) (BioRad, Richmond, CA) at 4°C. The pass-through fraction eluted with water was neutralized immediately with pyridine and the pH adjusted to between 6 and 7 (Nagasawa, et al., (1980a) Meth. Carbohyd. Chem., V 8, pp 287-289). After dialysis against water, the heparin pyridinium salt was collected by lyophilization. Partial N-desulfation, complete N-desulfation, and complete N- and O-desulfation, starting with 100 mg each of heparin pyridinium salts, were achieved by solvolysis in 10 ml of dimethylsulfoxide (ACS grade, Fisher Scientific, Fair Lawn, NJ.) containing water or methanol as described by Nagasawa, et al. *ibid* and Nagasawa, et al. (1980b) Meth. Carbohyd. Chem., V 8, pp 291-294. Reaction conditions were 10% water in dimethyl sulfoxide at 20°C for 1 h for partial N-desul-

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fation, 10% water in dimethyl sulfoxide at 80°C for 5 hr for complete N-desulfation, and 10% anhydrous methanol in dimethylsulfoxide at 80°C for 18 h for complete N- and O-desulfation. After these reactions, the mixtures were  
5 cooled and 1 M sodium hydroxide was added to adjust the pH to between 8.5 and 9.5; the mixtures were dialyzed against running tap water and then against distilled water.

N-acetylation of N-desulfated and N- and O-desulfated  
10 heparin was performed with acetic anhydride under alkaline conditions as follows: fifty milligrams of modified heparin were dissolved in 5 ml of 4.5 M sodium acetate plus 1.0 ml of methanol and added to five portions of 1.0 ml of acetic anhydride at 10 min intervals. After 1 hr  
15 incubation with occasional mixing, the reaction mixture was dialyzed against running tap water, then distilled water, and was finally lyophilized.

The N-resulfation reaction was performed by sulfation  
20 with a triethylamine sulfur trioxide complex prepared according to Cherniak and Davidson's (1964) method (J. Biol. Chem., V 239, pp 2986-2990). Complete N- and O-desulfated heparin (50 mg) was dissolved in 2 ml of 1.0 M sodium carbonate and added to 50 mg of triethylamine  
25 sulfur trioxide. The atmosphere was replaced with nitrogen, and the mixture was heated at 50°C for 24 h with occasional agitation. The resulfated heparin was dialyzed against running tap water, then distilled water, and finally lyophilized. Carboxyl-reduced heparin was  
30 prepared from sodium salt of porcine intestinal mucosa heparin as described by Taylor et al. (1976) (Meth. Carbohydr. Chem., V 7, pp 149-171) by use of sodium borohydride (Aldrich Chemical Co., Milwaukee, WI) and N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide-hydro-  
35 chloride (Fluka Chemical Co., Hauppauge, N.Y.).

Analytical Procedures. The homogeneity of the chemically modified heparins was assessed by cellulose acetate electrophoresis and high-speed gel-permeation chromatography. Electrophoresis was performed on a 7.6 x 7.6 cm Titan III Zip Zone cellulose acetate sheet (Helena Laboratories, Beaumont, TX) in 0.5 M/0.5 M pyridine-acetate buffer, pH 5.0 (Hata et al., (1979), Anal. Biochem., V 45, pp 412-469). Each 1 ul sample was applied to 2.0 cm wide stacking zone which was blotted with water before the sample application (Cappeletti et al., (1979) Anal. Biochem., V 99, pp 311-320). Electrophoresis was carried out under constant voltage (75V) for 45 min. During the run the sheet was immersed in petroleum ether cooled under ice. The cellulose acetate sheet was then stained with 0.1% toluidine blue O in 1% acetic acid and destained with 1% acetic acid.

High-speed gel permeation chromatography was performed as described previously (Irimura et al., (1983a) Anal. Biochem., V 130, pp 461-468) using a Constametric III (LDC-Milton Roy, Riviera Beach, FL) with a single 0.7 x 75 cm stainless steel column packed with Fractogel TSK HW-55(S). Elution was accomplished with 0.2 M sodium chloride at flow rates of 1.0 ml/min or 0.75 ml/min. Absorption at 210 nm was monitored for the analogs of chemically modified heparin. For the qualitative examination of radiolabeled HS-degradation products, each fraction corresponding to 30-sec elution was collected into plastic scintillation vials, and radioactivity in each vial was determined after the fraction was mixed with 3.0 ml of Hydrofluor (National Diagnostics, Somerville, NJ).

Source of Heparanase. Highly invasive mouse B16 melanoma (B16-BL6) cells were provided by Dr. I. J. Fidler, (M.D. Anderson Hospital, Houston, TX) and were

cultured as previously described (Irimura et al., (1983a) and Irimura et al. (1984)). Cell extracts were prepared in 5 mM Tris-HCl buffer, pH 7.4, containing 0.25 M sucrose, 50  $\mu$ M calcium chloride, 10  $\mu$ M phenylmethyl-  
5 sulfonylfluoride, and 0.2% Nonidet P-40 (Irimura et al., (1983)). The melanoma extracts were stored frozen at  $-80^{\circ}\text{C}$  and used as crude heparanase.

Radio-Labeling of HS.  $^{14}\text{C}$ - or  $^3\text{H}$ -heparan sulfate was  
10 prepared by chemical deacetylation and radioactive reacylation. Nine milligrams of bovine lung HS were dried with 0.1 mg of hydrazine sulfate over phosphorous pentoxide under vacuum at  $50^{\circ}\text{C}$  for 48 h. Anhydrous hydrazine (0.5 mg, Pierce Chemical, Rockford, IL) was  
15 added to the dried HS and the mixture heated in a tightly screwed tube under nitrogen atmosphere at  $100^{\circ}\text{C}$  for 1 h. After the reaction, the hydrazine was removed by repeated evaporation with toluene over sulfuric acid dessicant under vacuum conditions. To separate deacetylated HS from  
20 residual reagents and partial degradation products, the completely dried residue was dissolved in 0.5 ml of water and subjected to gel filtration on a  $0.8 \times 30$  cm column of BioGel P-10 and elution with distilled water. The void volume fraction was collected and lyophilized, and the  
25 yield by weight was about 60%. N-deacetylated HS was then N-acetylated with 50  $\mu\text{Ci}$  of  $^{14}\text{C}$ -acetic anhydride (10  $\text{mCi}/\text{mmole}$ : New England Nuclear, Boston, MA.) or 5  $\text{mCi}$   $^3\text{H}$ -acetic anhydride (400  $\text{mCi}/\text{mmole}$ : NEN) in 0.5 ml of 4 M sodium acetate for 18 hrs. N-acetylation was completed by  
30 addition of 0.1 ml of unlabeled acetic anhydride to the reaction mixture and incubation for 1 h.  $^{14}\text{C}$ - or  $^3\text{H}$ -HS was purified on a BioGel P-10 column as described above.

High-Performance Liquid Chromatography [HPLC] Assay  
35 for Heparanase. Fifty microliters of melanoma extract (equivalent to  $10^6$  cells) were mixed with chemically

- modified heparin (5 mg/ml in water), 50 ul of 4 X heparanase assay buffer (0.4 sodium phosphate buffer, pH 5.8, containing 0.4% Triton X-100, 0.6 M sodium chloride, and 0.4 % sodium azide) and about 3000 cpm of  $^{14}\text{C}$ -HS.
- 5 Incubation was performed at 37°C with continuous gentle mixing for 6 h. The reaction mixture was placed on ice, 20 ul of 50% trichloroacetic acid was added, and incubation continued on ice for 10 min. After centrifugation at 9000 x G for 5 min in a Microfuge B (Beckman
- 10 Instruments, Irvine, CA), the supernatant was injected into the gel-permeation chromatography system and analyzed as described above (Irimura et al., (1983a)).

- Preparation of Solid-phase Substrates for Heparanase  
15 and Inhibitor Assays. For the solid-phase heparanase assay,  $^3\text{H}$ -HS was aminated at the reducing terminal with 2 M ammonium acetate in the presence of 0.4 M sodium cyanoborohydride in 50% methanol, at 50°C for 6 days. Aminated  $^3\text{H}$ -HS was purified by gel filtration as described
- 20 above, and the resulting solution was diluted to 0.1 M in sodium carbonate. To  $10^6$  cpm of aminated  $^3\text{H}$ -HS, 1.0 ml of Affi-Gel 15 (Bio Rad) gel beads was added after they were washed with isopropanol and chilled water. The coupling reaction was continued at 4°C for 48 h with continuous
- 25 agitation. The gel beads were then reacted with 0.1 M glycine monomethyl ester dissolved in 0.1 M sodium carbonate for 1 h at room temperature and then washed with 4 M sodium chloride repeatedly to remove noncovalently attached  $^3\text{H}$ -HS from the beads.

30

- Solid-phase Assays for Heparanase and Heparanase  
Inhibitors.  $^3\text{H}$ -HS-agarose was suspended in Dulbecco's phosphate buffered saline (DPBS) at about 20% (v/v). The incubation conditions for the solid-phase assay were
- 35 identical to those of the HPLC assay, except that 75 ul of the  $^3\text{H}$ -HS-agarose suspension was used instead of the HS so

that the incubation mixture consisted of B16 melanoma extract, chemically modified heparins, 4X heparanase assay buffer, and  $^3\text{H}$ -HS-agarose suspension. After incubation, the reaction mixture was placed on ice, chilled 5% trichloroacetic acid (50  $\mu\text{l}$ ) added, the mixture incubated for 10 min, and centrifuged. Radioactivity in the supernatant and the pellet was determined separately after mixing with Hydrofluor.

10        Experimental Metastatic Lung Colonization of Melanoma Cells. Male C57BL/6 mice 4 to 6 weeks old were obtained from Charles River, Inc. (Kingston, MD) and quarantined for 2 weeks. Animals were fed normal rodent chow and unchlorinated spring water. B16-BL6 cells were grown to subconfluence, detached from plastic dishes by incubating in 2 mM EDTA, 0.14 M NaCl and 10 mM sodium phosphate-buffer, pH 7.4, for 5-10 min, and suspended in a 1:1 mixture of Dulbecco's modified minimum essential medium and Ham's F12 medium. The cells were incubated with heparin, N-acetylated N-desulfated heparin, N-resulfated N and O-desulfated heparin or carboxy-reduced heparin (each 0.5 mg/ml in the media described above) at 4°C for 2 hrs. Treated or untreated cells ( $5 \times 10^4/0.1 \text{ ml}$ ) were injected intravenously to each mouse. Mice were killed 20 days later and autopsied. The numbers of pulmonary tumor nodules were counted after the lung was perfused via the trachea with 4% formalin in Dulbecco's phosphate buffered saline. Extrapulmonary tumor formation was assessed in each animal and recorded.

30

## EXAMPLE 2

## CHEMICALLY MODIFIED HEPARINS

5        Chemical Modifications. Porcine intestinal mucosal  
heparin was chemically modified as schematically shown by  
the representative structures in Figure 1. When the  
electrophoretic mobilities of these substances in  
pyridine/acetate buffer on cellulose acetate sheets were  
10 compared (Figure 2), all of the chemical modified heparins  
migrated more slowly than intact heparin under the  
electrophoretic condition used. N- and O-desulfated  
heparins remained at the top of stacking gel and did not  
stain intensely with Toluidine blue O. The migration  
15 distances of the modified heparins were, in order from  
shortest to longest: N- and O-desulfated heparin, N-  
acetylated N- and O-desulfated heparin, N-desulfated  
heparin, N-resulfated N- and O-desulfated heparin, N-  
acetylated N-desulfated heparin, partially N-desulfated  
20 heparin, and carboxy-reduced heparin. High-speed gel-  
permeation chromatography of these substances was  
performed as described in Example 1 (Figure 3). A slight  
change in the apparent molecular size was observed,  
probably as a result of detachment and reattachment of the  
25 relatively bulky side groups to the heparin chain.  
Degradation products were not observed in any of the  
modified heparin preparations as determined by high speed  
gel permeation chromatography.

## EXAMPLE 3

HPLC ASSAY OF HEPARANASE INHIBITORY  
ACTIVITIES OF CHEMICALLY MODIFIED HEPARINS

5 Intact heparin, N-desulfated heparin, N- and O-  
desulfated heparin, and N-sulfated N- and O-desulfated  
heparin (4 mg/ml dissolved in water) were mixed with 50  $\mu$ l  
of crude heparanase and  $^{14}$ C-HS as described above. The  
10 elution profiles of the radioactivity on high-speed gel-  
permeation chromatography are shown in Figure 4. The  
elution profile of  $^{14}$ C-HS incubated with heat-inactivated  
(100°C for 5 min) heparanase was identical to that of  
untreated  $^{14}$ C-HS, which eluted at the position corres-  
15 ponding to an approximate  $M_r$  of 34,000. After incubation  
with heparanase, the average apparent  $M_r$  decreased to  
6,000. In the presence of heparin, no degradation was  
observed. N-desulfated heparin or N- and O-desulfated  
heparin failed to inhibit degradation of HS by heparanase.  
20 After N-resulfation of N- and O-desulfated heparin, the  
heparanase inhibitory activity was partially restored.  
O-sulfate groups on the 3 or 6 position of glucosamine, as  
well as on the 2 position of iduronic acid, seem less  
essential to heparanase inhibition, provided that the  
25 amino groups are completely resulfated.

## EXAMPLE 4

## 30 SOLID-PHASE ASSAY FOR HEPARANASE

Since heparanase is an endoglycosidase that produces  
relatively large fragments of HS, rapid isolation of the  
fragmented HS from intact HS is necessary for the quanti-  
35 tative assay. Therefore, HS chains were immobilized at  
their ends to a solid-phase support, such as small beads.

First,  $^{35}\text{S}$ -HS (purified from the subendothelial matrix in cell culture (Wang et al., (1985) Eur. J. Biochem., V 153, pp 125-130) were immobilized onto Affi-Gel 15, since this HS preparation contained amino acid residues at its

5 reducing terminal end. With use of this substrate, however, the proportion of  $^{35}\text{S}$ -labeled materials released from the beads by crude heparanase was negligible, and this method was not found likely to be useful. Next, chemically labeled and modified HS was immobilized on

10 beads. Deacetylation of HS was achieved by hydrazinolysis. Lower temperatures and shorter reaction times than the usual hydrazinolysis reactions of glyco-proteins (Fukuda et al., (1976) J. Biochem., V 80, pp 1223-1232); Irimura et al., (1981) Biochem, V 20, pp 560-

15 566) were chosen because of possible cleavage of uronosyl-glucosaminide through these amino groups. Resultant N-deacetylated heparin was labeled by N- $^{14}\text{C}$ - or  $^3\text{H}$ -acetylation. It appeared to be important to subsequently block all free amino groups by acetylation with

20 non-radioactive acetic anhydride, which was done. Radio-labeled HS was aminated exclusively at its reducing terminal by reductive amination, and coupled to Affi-Gel 15 linkage under alkaline conditions. The proportion of labeled HS coupled to agarose beads fluctuated between 50

25 and 80% calculated from the amount of material used for the amination reaction. As a heparanase substrate, partially N-desulfated and N-  $^3\text{H}$ - or  $^{14}\text{C}$ -acetylated HS are also as useful as deacetylated and reacetylated HS. Partial N-desulfation of HS was achieved by the same

30 reaction conditions used for partial N-desulfation of heparin described under Materials and Methods.



## EXAMPLE 5

HEPARANASE INHIBITION BY CHEMICALLY MODIFIED  
HEPARINS AS MEASURED BY A SOLID-PHASE ASSAY

5

The dose response curves of the inhibitory effects of chemically modified heparins are shown in Figure 5. The use of i) intact, ii) N-desulfated, iii) N- and O-desulfated and iv) N-resulfated, N- and O-desulfated heparin produced results consistent with those of the HPLC assay. N-desulfated and N- and O-desulfated heparin failed to show any inhibitory activity, whereas partial restoration of activity was obtained by the addition of sulfamino groups (Figure 5g). Interestingly, when the exposed amino groups formed by N-desulfation were acetylated, heparanase inhibitory activity was partially restored (Figure 5d). N-Acetylation of N- and O-desulfated heparin did not, however, restore the inhibitory activity (Figure 5f). These results indicated that sulfamino groups, although they affected heparanase inhibition, were not essential for it. Removal of sulfamino groups, but with intact O-sulfate groups of heparin resulted in inhibitory activity, provided that the exposed amino groups are blocked by acetylation. Carboxy-reduced heparin was shown to possess weaker inhibitory activity than heparin, which indicated that heparin carboxy groups were necessary but not essential, for the full inhibitory activity. Similar observations have been described concerning heparin's stimulatory activity on the growth of smooth muscle cells (Castellot et al., (1984) J. Cell. Physiol., V 120, pp 315-320).

## EXAMPLE 6

## MODIFIED HEPARINS AS ANTICOAGULANTS

5       Anticoagulation activities of N-acetylated N-desulfated heparin, N-resulfated N- and O-desulfated heparin, and carboxyl-reduced heparin were measured by the USP standard assay. The anticoagulation activities of these three compounds were each less than 1% of the  
10   anticoagulation activity of unmodified heparin.

## EXAMPLE 7

15                   EFFECTS OF MODIFIED HEPARINS ON  
                    EXPERIMENTAL BLOOD-BORNE LUNG  
                    COLONIZATION OF B16 MELANOMA IN MICE

20       As shown in Table 1, the number of visible melanoma colonies in lung 20 days after the injection of the tumor cells was significantly reduced by preincubation of the cells with intact or chemically modified heparin. The effect of intact heparin was greater, probably because additional factors such as inhibition of melanoma-induced  
25   platelet aggregation were involved. Since these three chemically modified heparins do not possess anticoagulation activity, the inhibition of melanoma lung colonization appeared to be due to inhibition of melanoma heparanase.

TABLE 1

THE EFFECTS OF CHEMICALLY-MODIFIED HEPARINS ON BLOOD-BORNE LUNG  
COLONIZATION OF B16-BL6 MELANOMA CELLS IN MICE

| Treatment <sup>a</sup>                    | Exp 1 (9 mice/group)<br>Number of colonies (median) | Exp 2 (9 mice/group)<br>Number of colonies (median) |
|---|---|---|
| None                                      | 8, 20, 28, 33, 75, 85, 106,<br>116, 193, (75)       | 0, 1, 26, 48, 75, 163, 193,<br>200+, 200+, (75)     |
| Heparin                                   | 0, 1, 8, 9, 16, 18, 21, 82,<br>174, (16)            | 0, 0, 0, 0, 0, 1, 1, 2, 12, (0)                     |
| N-acetylated-N-de-<br>sulfated heparin    | 11, 19, 22, 23, 43, 89, 109,<br>199, (43)           | 0, 1, 2, 3, 5, 5, 25, 37,<br>200+, (5)              |
| N-resulfated-N-, O-<br>desulfated heparin |   | 0, 0, 2, 5, 8, 13, 20, 90,<br>200+, (8)             |
| Carboxy-reduced<br>heparin                | 0, 15, 25, 25, 29, 36, 45, 46,<br>53, (29)          | 7, 13, 42, 49, 51, 55, 58, 89,<br>120, (51)         |

-34-

<sup>a</sup>The cells were incubated with chemically modified heparins (500 ug/ml) at 4°C for 2 hr before injection.

## EXAMPLE 8

EFFECTS OF MODIFIED HEPARINS ON THE DEGRADATION OF LUNG  
CAPILLARY ENDOTHELIAL MATRIX BY MELANOMA CELLS IN VITRO

5           Effects of chemically modified heparins on the  
release of  $^{35}\text{S}$ -labeled polysaccharides from the extra-  
cellular matrix of mouse lung endothelial cells were  
studied in vitro. The endothelial cells isolated from  
10 mouse lung capillary were grown in the presence of  $^{35}\text{S}$ -  
sulfate, and the extracellular matrix-like material was  
isolated on multiwell plastic tissue culture plates by  
hypotonic lysis of the cells. The matrices (1 cm  
diameter) were incubated with mouse BL6-BL6 melanoma at  
15 37°C for 18 hours. The released radioactivity was  
counted.

          As shown in the Figure 6, N-resulfated- N-, O-  
desulfated heparin and carboxyl-reduced heparin  
20 effectively blocked the degradation of sulfated molecules,  
most likely heparan sulfate proteoglycans, by melanoma  
cells. Heparin apparently induced spontaneous release of  
 $^{35}\text{S}$  labeled macromolecules, while such effects were  
minimal with modified heparins.

25

## EXAMPLE 9

EFFECTS OF MODIFIED HEPARINS ON THE GROWTH  
OF B16 MELANOMA CELLS IN TISSUE CULTURE

30

          In order to assess a possibility that the effects of  
modified heparins on the blood-borne lung colonization of  
B16 melanoma cells were due to their direct toxic effects  
35 on the cells, the growth of B16 melanoma in the presence  
or absence of modified heparins were examined in vitro.

B16-BL6 melanoma cells were seeded in multiwell tissue culture plates at a density 10,000 per each 1 cm well in the presence or absence of 5% fetal bovine serum. Six hours later, modified heparins were added, then 24 hours later, <sup>3</sup>H thymidine was added to a final concentration 5 uCi/ml. After further 24 hour incubation, trichloroacetic acid-insoluble materials were collected, dissolved in 1 M sodium hydroxide solution, neutralized and counted on a liquid scintillation counter.

10

Figure 7 shows typical data demonstrating that there are no significant effects on the incorporation of <sup>3</sup>H-thymidine to B16 cells shown by unmodified heparin N-acetylated-N-desulfated heparin, N-resulfated- N-, O-desulfated heparin or carboxyl-reduced heparin. These data indicated that the in vivo effects of these compounds were due to the influence on the tumor cell-host interactions.

20

## EXAMPLE 10

25 EFFECTS OF MODIFIED HEPARINS ON THE ADHESION OF B16 MELANOMA CELLS TO THE MONOLAYER AND TO THE EXTRACELLULAR MATRIX OF LUNG CAPILLARY ENDOTHELIAL CELLS

One of the most critical steps in the tumor cell-microvascular endothelium interaction during metastatic lung colonization of melanoma cells is the adhesive interaction of melanoma cells with endothelial cells. The effects of modified heparins on the adhesion of B16 melanoma to the cell monolayer and to the extracellular matrix of lung capillary endothelial cells were studied in vitro. The endothelial cells were grown in tissue culture and the extracellular matrices were isolated as described in Example 8. Figure 8(a) shows the time course of the

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adhesion of B16-BL6 cells previously treated with modified heparins to the monolayer of endothelial cells, in the conteneous presence of modified heparins. None of these compounds, including intact heparin, significantly altered the time course of the adhesion of B16-BL6 cells to the endothelial cell monolayer. Prior treatment of endothelial cells for 2 hrs with these compounds also resulted in no significant alteration in the adhesion (Figure 8(b)).

10

## EXAMPLE 11

15 EFFECTS OF MODIFIED HEPARINS ON ADHESION  
TO AND INVASION OF ORGAN-CULTURED  
LUNG TISSUES BY MELANOMA CELLS IN VITRO

The effects of these glycosaminoglycan derivates on organ-specific adhesion and invasion of B16 melanoma cells were studied by using primary organ culture techniques in vitro. Minced lung tissues (0.5-1.0 cubic mm) from C57BL/6 mice were placed in glass vials and incubated in HEPES-buffered Dulbecco's modified minimum essential media containing 10% fetal bovine serum and 50 ug/ml gentamycin under continuous gyration at 66 rpm. After the medium was changed to a fresh medium without gentamycin, B16-F10 melanoma cells were added at a final concentration of 100,000/ml with or without various modified heparins. After various incubation times, the tissues were fixed in buffered formalin and processed for light microscopy. The adhesion and invasion of the tumor cells were examined on 4 mm thick sections stained with hematoxylin and eosin.

As shown in Figure 9, the number of melanoma cells attaching to the surface of minced lung tissues was significantly affected by the compounds at the early

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stages of incubation (6 hrs), but little effect was present after 18 hrs. Heparin and modified heparins inhibited invasion of B16 melanoma cells to the lung tissues, as shown in Figure 10. These data clearly indicated that these compounds possess capacity to block lung invasion of B16 melanoma cells.

## EXAMPLE 12

10

IN VIVO INHIBITION BY HEPARIN  
DERIVATIVES OF METASTATIC COLONIZATION

Chemically modified heparins were tested in mice as inhibitors of blood-borne lung colonization by melanoma cells. Male C57BL/6 mice 4 to 6 weeks old were obtained from Charles River, Inc. and quarantined for 2 weeks. The mice were intravenously inoculated with  $5 \times 10^4$  B16-BL melanoma cells. Treated mice were intravenously injected with 0.1 ml each of heparin or chemically modified heparin (5.0 mg/ml) 4 hr before and 20 hr after inoculation with the melanoma cells. The chemically modified heparins used were: N-acetylated N-desulfated heparin; carboxyl-reduced heparin; and N-resulfated N, O-desulfated heparin.

25

Mice were killed 20 days after tumor inoculation and autopsied. The numbers of pulmonary tumor colonies were counted after the lungs were perfused via the trachea with 4% formalin in Dulbecco's phosphate-buffered saline. The results of these manipulations are shown in Table 2. As may be seen in Table 2, heparin as well as the glycosaminoglycan derivatives substantially devoid of anticoagulation activity all impeded the establishment of metastatic tumor colonies in the lungs. Although heparin itself inhibited formation of lung metastases, the

35



carboxyl-reduced heparin was most effective in this particular manner.

TABLE 2

EFFECTS OF CHEMICALLY MODIFIED HEPARINES  
ON BLOOD-BORNE LUNG COLONIZATION OF B16  
MELANOMA CELLS IN SYNGENEIC MICE

| Treatment                            | Number of lung colonies      | Median |
|--------------------------------------|------------------------------|--------|
| None                                 | 3,>150,>150,>150,>150,>150   | >150   |
| Heparin                              | 19,20,29,30,40,99,100        | 30     |
| N-acetylated N-desulfated heparin    | 4,26,28,50,60,>150,>150,>150 | 60     |
| Carboxyl-reduced heparin             | 0,0,1,3,6,6,60,61            | 6      |
| N-resulfated, N,0-desulfated heparin | 0,1,28,28,30,36,38,>150,>150 | 36     |

\* \* \* \* \*

5

Changes may be made in the operation and arrangement of the various elements, substances and procedures described herein without departing from the concept and scope of the invention as defined in the following claims.

## CLAIMS:

1. A method for impeding the formation of tumor metastases in a host comprising:

5

providing a glycosaminoglycan derivative which is substantially devoid of anticoagulation activity and is an effective inhibitor of heparanase activity; and

10

exposing host-borne tumor cells to the glycosaminoglycan derivative by parenteral administration of the glycosaminoglycan derivative to the host.

15

2. A method for inhibiting the invasiveness of tumors in a host, the method comprising inhibiting heparanase activity of said tumors by administration to the host of an amount of heparanase inhibitor which is a glycosaminoglycan derivative substantially devoid of anticoagulant activity.

20

3. The method of claim 2 wherein the amount is between about 30 mg/day and 250 mg/day.

25

4. The method of claim 2 wherein the amount is between about 30 mg/day and 100 mg/day.

30

5. A composition of matter comprising heparin derivatized to substantially remove its anticoagulation activity while leaving at least a substantial portion of its heparanase-inhibitory activity intact, said

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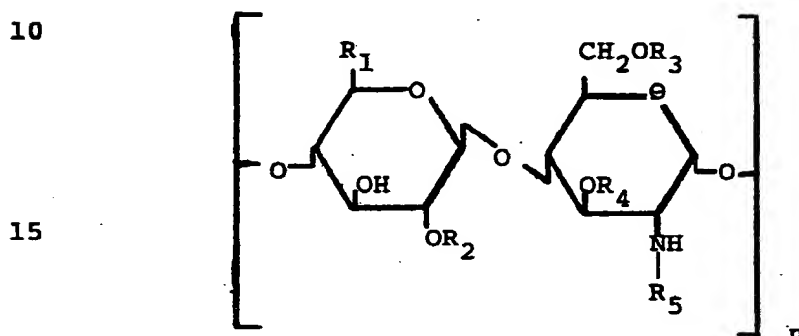
derivatization including the step of at least partial N-desulfation or carboxyl-reduction.

- 5 6. The composition of claim 5 wherein the derivatization includes the step of at least partial N-desulfation and the additional step of N-acetylation is added.
- 10 7. A method for inhibiting the spreading of metastatic tumor cells subsequent to or simultaneous with surgical removal of a primary tumor from a host, the method comprising parenterally administering to said host an effective amount of a glycosaminoglycan derivative sub-
- 15 stantially without anticoagulant activity but with heparanase inhibitory activity.
8. A composition comprising a heparin derivative
- 20 produced by: at least partial N-desulfation of heparin followed by N-acetylation; at least partial N- O-desulfation of heparin followed by N-resulfation; or carboxyl-reduction of heparin.
- 25 9. A formulation for impeding the spread of cancer cells, the formulation comprising:
- 30 an effective amount of a glycosaminoglycan derivative capable of inhibiting the activity of glycosaminoglycan endohydrolases and being substantially devoid of anticoagulant activity; and

a pharmaceutically acceptable carrier.

10. The formulation of claim 9 wherein the glycosamino-  
5 glycan derivative comprises sulfamino or O-sulfate groups.

11. A glycosaminoglycan derivative having the formula:



Where

20

$R_1$  is  $-\text{COOH}$  or  $-\text{CH}_2\text{OH}$  and the configuration of the carbon atom to which  $R_1$  is bound is  $\underline{D}$  or  $\underline{L}$ ;

$R_2$  is  $-\text{H}$  or  $-\text{SO}_3^-$ ;

25

$R_3$  is  $-\text{H}$  or  $-\text{SO}_3^-$ ;

$R_4$  is  $-\text{H}$  or  $-\text{SO}_3^-$ ;

$R_5$  is  $-\text{H}$ ,  $-\text{SO}_3^-$  or  $-\text{CO}-\text{CH}_3$ ;

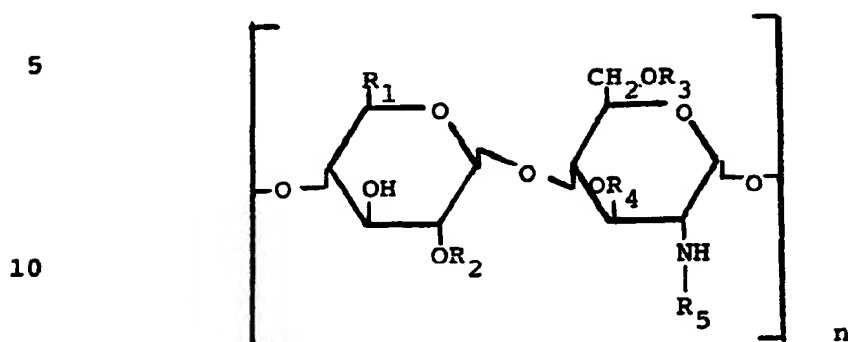
$n$  is 3 to 30; and

each of the terminal monomeric units

30

is a monomeric repeating unit with the terminal oxygen atom being bound to a blocking group.

12. A glycosaminoglycan derivative having the formula:



15 Where

$R_1$  is  $-\text{COOH}$  or  $-\text{CH}_2\text{OH}$  and the configuration of the carbon atom to which  $R_1$  is bound is  $\underline{D}$  or  $\underline{L}$ ;

20

$R_2$  is  $-\text{H}$  or  $-\text{SO}_3^-$ ;

$R_3$  is  $-\text{H}$  or  $-\text{SO}_3^-$ ;

$R_4$  is  $-\text{H}$  or  $-\text{SO}_3^-$ ;

$R_5$  is  $-\text{SO}_3^-$  or  $-\text{CO}-\text{CH}_3$ ; and

25

$n = 3-30$ ;

provided that: when  $R_1$  is  $-\text{CH}_2\text{OH}$ , at least 50% of  $R_2$ ,  $R_3$  and  $R_5$  is  $-\text{SO}_3^-$ ;

30

when  $R_1$  is  $-\text{COOH}$ , and  $R_2$ ,  $R_3$  and  $R_4$  are  $-\text{H}$ ,  $R_5$  is  $-\text{SO}_3^-$ ;

when  $R_1$  is  $-\text{COOH}$ , and at least 50% of  $R_2$ ,  $R_3$  and

$R_4$  is  $-\text{SO}_3^-$ ,  $R_5$  is  $-\text{CO}-\text{CH}_3$ ; and

5 each of the terminal monomeric units is a repeating monomeric unit having a terminal oxygen atom bound to a blocking group.

10 13. A composition of matter comprising a glycosaminoglycan derivative capable of inhibiting the activity of a glycosaminoglycan endohydrolase and being substantially devoid of anticoagulant activity.

15

14. The composition of claim 13 wherein the glycosaminoglycan is heparin, hyaluronic acid, chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, keratan sulfate or heparan sulfate.

20

15. The formulation of claim 9 wherein the glycosaminoglycan derivative is described in claim 11.

25

16. The formulation of claim 9 wherein the glycosaminoglycan derivative is described in claim 12.

30

17. The glycosaminoglycan derivative of claim 11, 12 or 13 described further as having a molecular weight between about 1,000 and about 15,000.

35

18. The glycosaminoglycan of claim 17 wherein the molecular weight is between about 10,000 and about 12,500.



19. A method for producing a cancer-inhibiting substance comprising:

5 identifying a material which inhibits heparanase activity and has anticoagulation activity; and

10 chemically altering this material to substantially remove anticoagulation activity while enhancing or at least not removing heparanase-inhibitory activity.

20. A method for producing a cancer-inhibiting substance comprising:

15 obtaining a glycosaminoglycan which substantially neither has anticoagulant activity nor inhibits heparanase; and

20 chemically altering the glycosaminoglycan by a process comprising sulfation of amino groups, to produce a glycosaminoglycan derivative having heparanase activity but being substantially devoid of anticoagulant activity.

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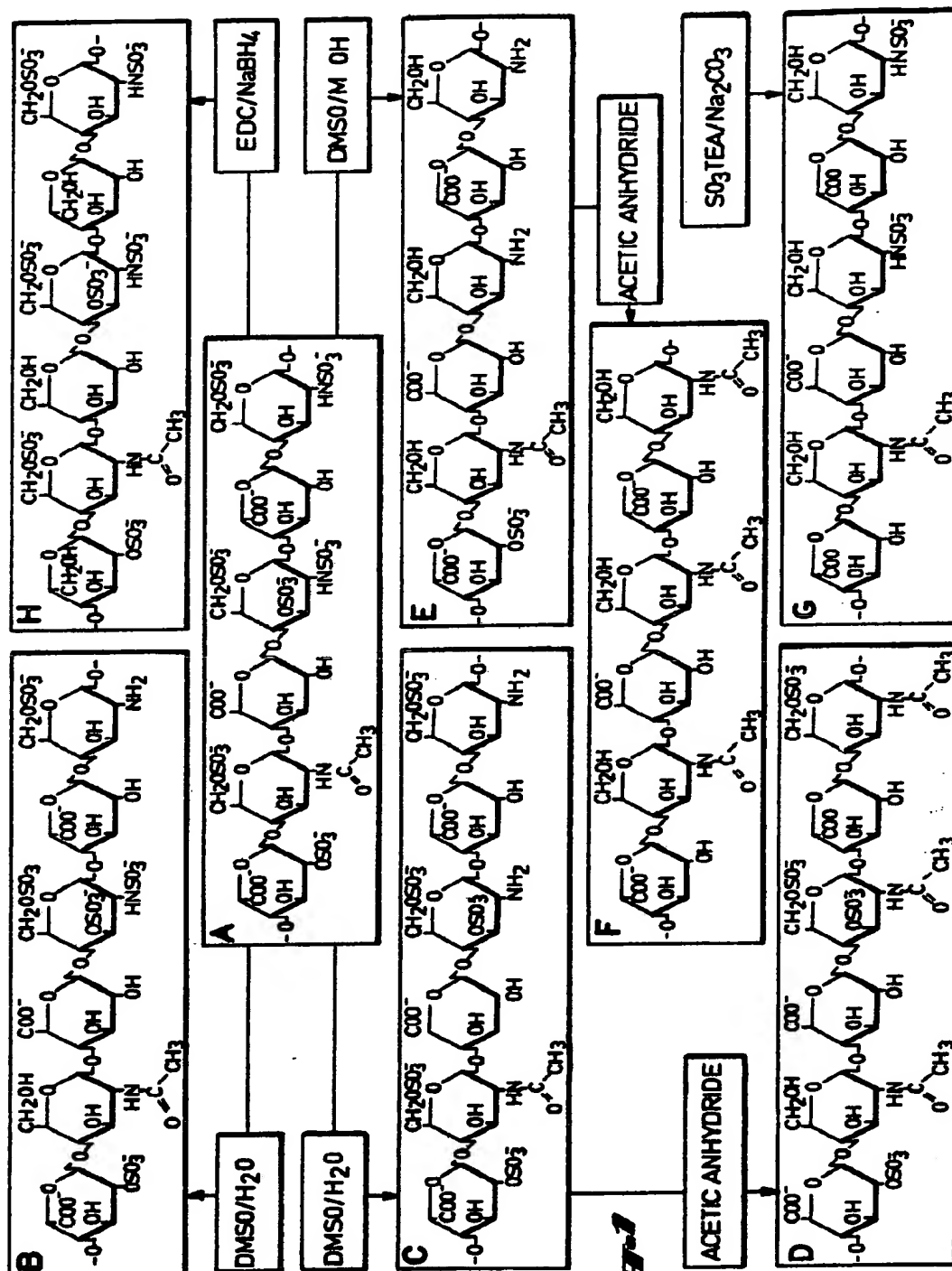
21. The method of claim 19 wherein the material is a sulfated glycosaminoglycan.

30

22. The method of claim 19 wherein the material is heparin.

35 23. A cancer-inhibiting substance produced by the method of claim 19, 20, 21 or 22.

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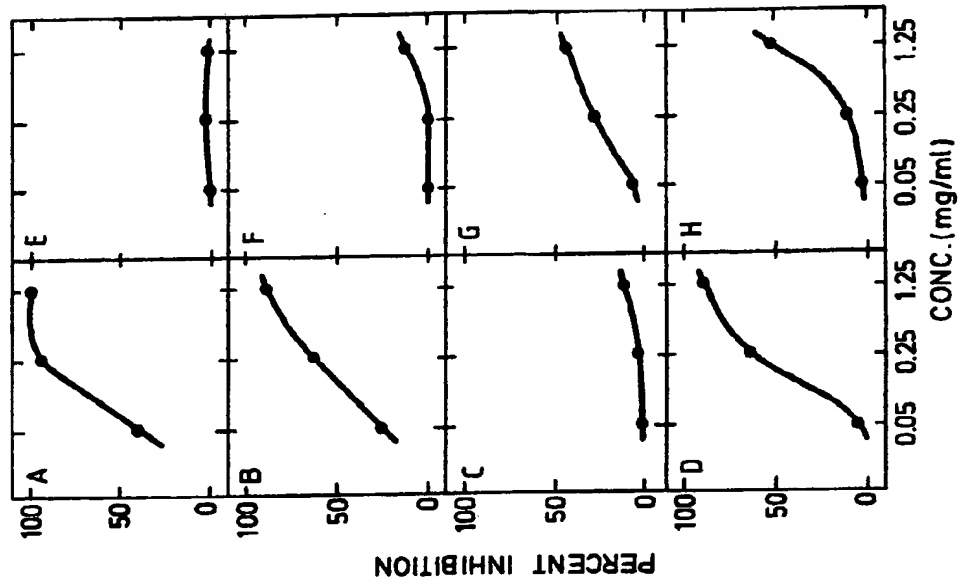


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Fig. 1

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**Fig. 5**



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**Fig. 2**

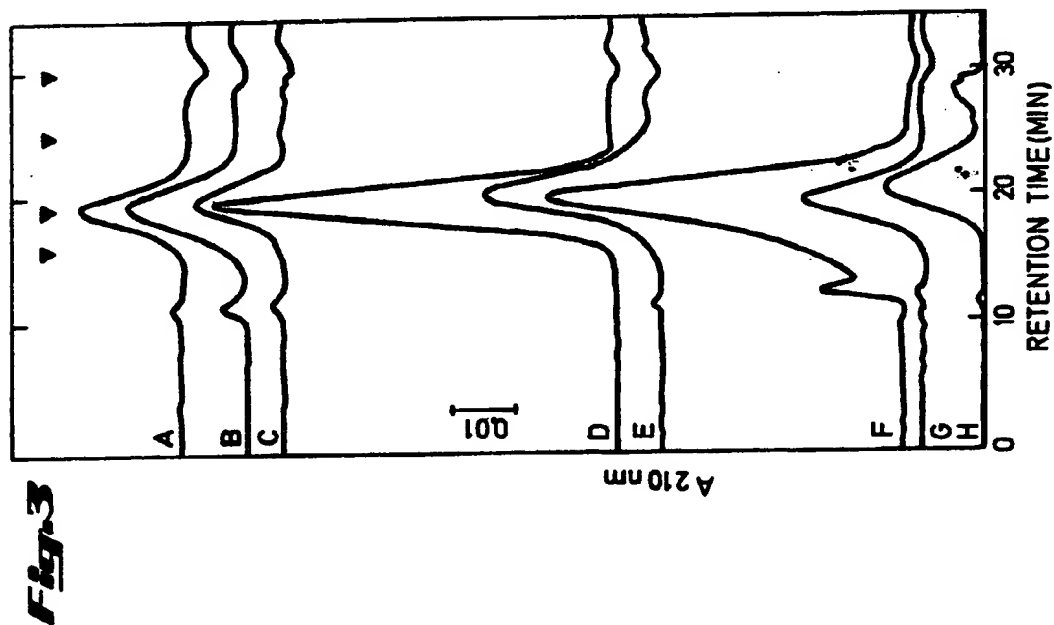
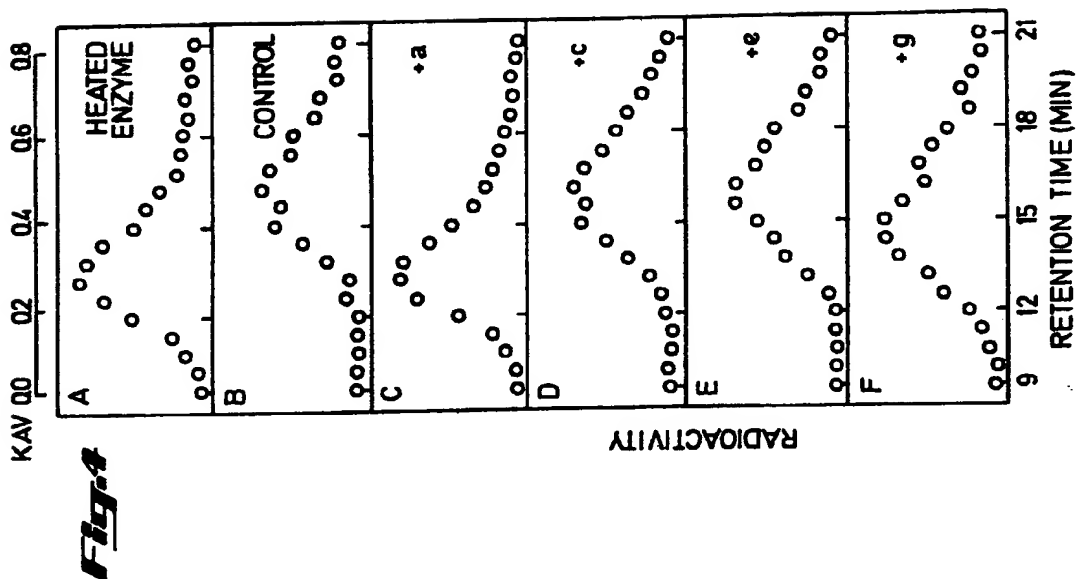


ORIGIN

H. CARBOXYL REDUCED  
G. N-RESULFATED NO-DESULFATED  
F. N-ACETYLATED NO-DESULFATED  
E. NO-DESULFATED  
D. N-ACETYLATED N-DESULFATED  
C. COMPLETELY N-DESULFATED  
B. PARTIALLY N-DESULFATED  
A. INTACT

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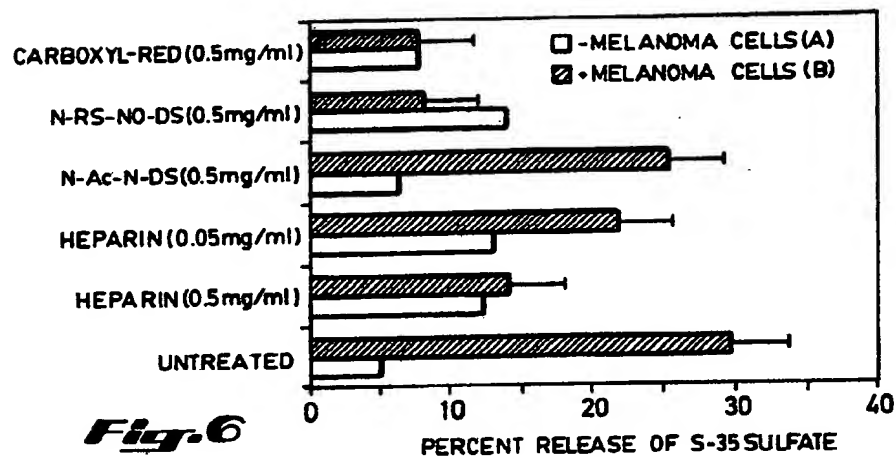
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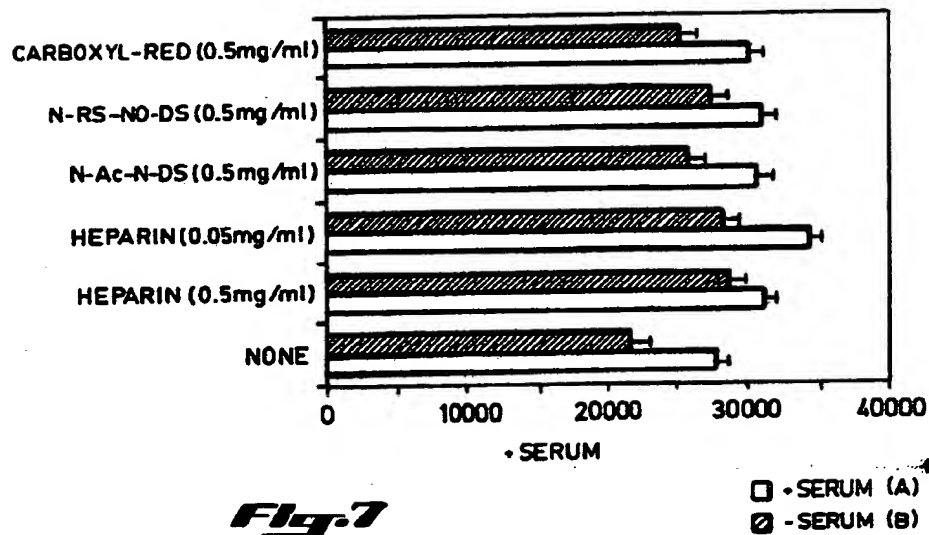
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## EFFECTS OF MODIFIED HEPARINS ON ENDOTHELIAL MATRIX DEGRADATION

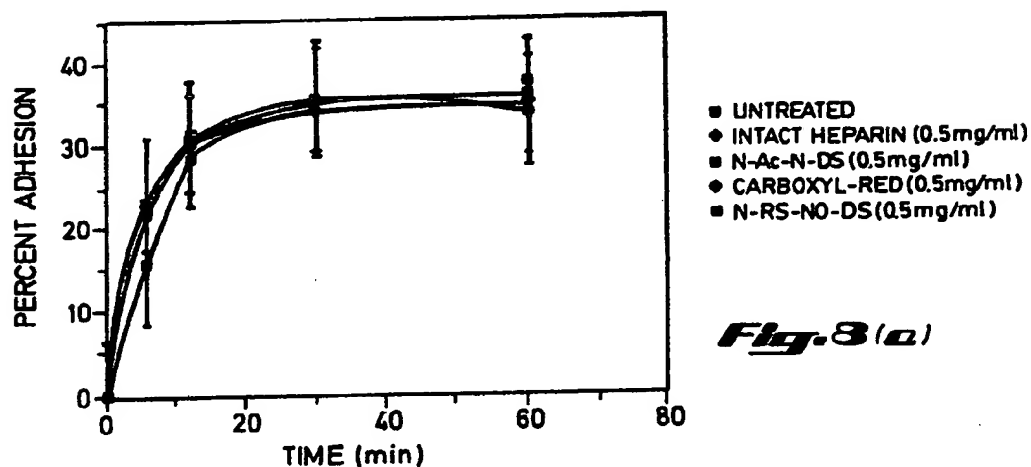
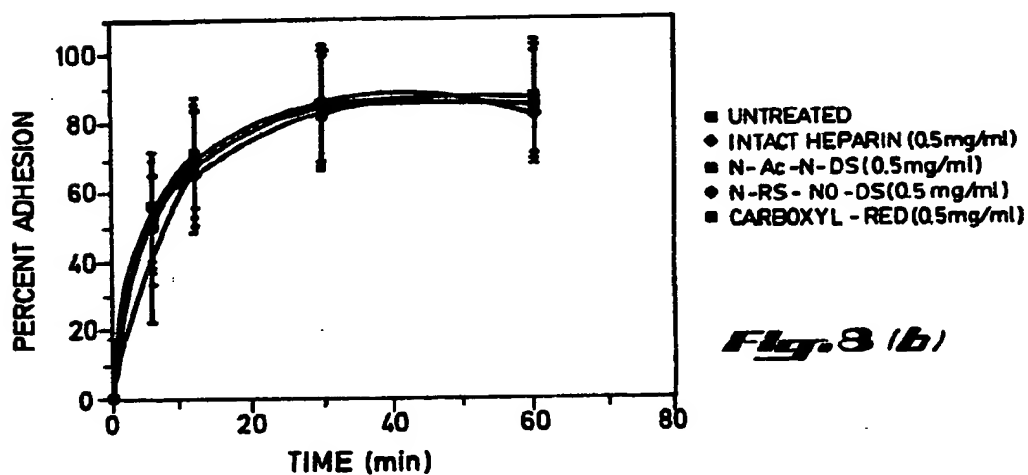


## EFFECTS OF MODIFIED HEPARINS ON THE GROWTH OF B16-BL6 MELANOMA CELLS



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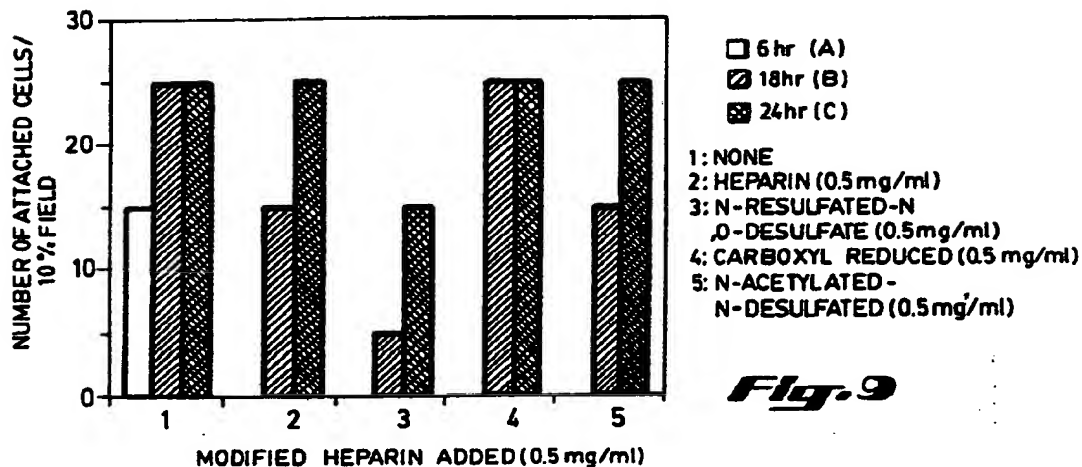
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EFFECTS OF MODIFIED HEPARIN-TREATMENT OF MELANOMA CELLS  
ON THEIR ADHESION TO ENDOTHELIAL MONOLAYER**Fig. 8 (a)**ADHESION OF MELANOMA CELLS TO ENDOTHELIAL CELLS  
PREVIOUSLY TREATED WITH MODIFIED HEPARINS**Fig. 8 (b)**

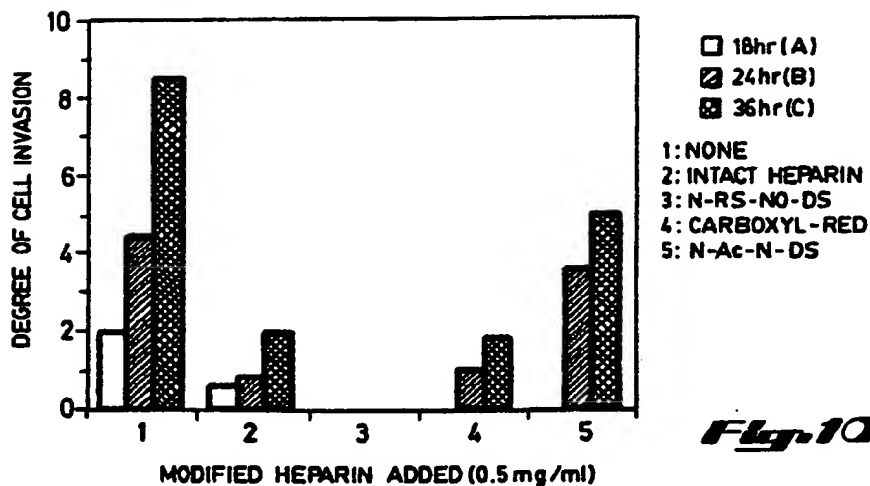
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## INHIBITION OF MELANOMA ADHESION TO LUNG TISSUE

**Fig. 9**


## INHIBITION OF MELANOMA INVASION TO LUNG

**Fig. 10****SUBSTITUTE SHEET**

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 87/02035

|  |  |   |
|--|--|---|
| <b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) *<br>According to International Patent Classification (IPC) or to both National Classification and IPC  |  |   |
| IPC <sup>4</sup> :      C 08 B 37/10; A 61 K 31/725  |  |   |
| <b>II. FIELDS SEARCHED</b>   |  |   |
| Minimum Documentation Searched <sup>7</sup>  |  |   |
| Classification System  | Classification Symbols   |   |
| IPC <sup>4</sup>   | C 08 B; A 61 K   |   |
| Documentation Searched other than Minimum Documentation<br>to the extent that such Documents are included in the Fields Searched <sup>8</sup>  |  |   |
|  |  |   |
| <b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>  |  |   |
| Category <sup>10</sup>   | Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>   | Relevant to Claim No. <sup>13</sup>   |
| Y  | EP, A, 0140781 (DROPIC) 8 May 1985<br>see page 10, lines 23-36; claims;<br>pages 14-15<br>--   | 5,6,8-23  |
| A  | Patent Abstracts of Japan, volume 10,<br>no. 139 (C-348)(2196), 22 May 1986,<br>& JP, A, 6117 (SEIKAGAKU KOGYO K.K.)<br>6 January 1986<br>see the whole abstract<br>--   |   |
| A  | Carbohydrate Research, volume 46, 1976,<br>Elsevier Scientific Publishing Co.,<br>(Amsterdam, NL),<br>Y. Inoue et al.: "Selective<br>N-desulfation of heparin with<br>dimethyl sulfoxide containing<br>water or methanol", see pages 87-95<br>-- |   |
| Y  | Science, volume 221, 19 August 1983,<br>J. Folkman et al.: "Angiogenesis<br>inhibition and tumor regression<br>caused by heparin or a heparin<br>fragment in the presence of ./.   |   |
| <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: <sup>14</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"G" document member of the same patent family</p> </div> </div> |  |   |
| <b>IV. CERTIFICATION</b>   |  |   |
| Date of the Actual Completion of the International Search  |  | Date of Mailing of this International Search Report   |
| 18th December 1987   |  | 25 JAN 1988   |
| International Searching Authority  |  | Signature of Authorized Officer   |
| EUROPEAN PATENT OFFICE   |  |  <b>P.C.G. VAN DER PUTTEN</b> |



| III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET) |   |                      |
|--|---|----------------------|
| Category *   | Citation of Document, with indication, where appropriate, of the relevant passages  | Relevant to Claim No |
|  | Cortisone", pages 719-725<br>see page 719, last paragraph and<br>page 720   | 5,6,8-23             |
|  | --  |                      |
| X  | Journal of Cellular Biochemistry,<br>supplement 9A, Abstracts of the<br>14th Annual Meeting, 12 January -<br>6 February 1985, Alan R. Liss, Inc.,<br>(New York, US),<br>Tatsuro Irimura et al.: "Chemically<br>modified heparins as inhibitors of<br>heparan sulfate endoglycosidase<br>from metastatic tumor cells",<br>see page 148, abstract 0369                      | 5,6,8-23             |
| X  | Journal of Cellular Biochemistry,<br>supplement 10A, Abstracts of the<br>15th Annual Meeting, 20 January -<br>15 February 1986, Alan R. Liss, Inc.,<br>(New York, US),<br>Tatsuro Irimura et al.: "Chemical and<br>biological characterization of semi-<br>synthetic inhibitors against<br>metastatic melanoma cell-derived<br>heparanase", see page 53, abstract<br>A141 | 5,6,8-23             |
|  | -----   |                      |

**FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET**

**V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE <sup>1</sup>**

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers 0)..... because they relate to subject matter not required to be searched by this Authority, namely:

0) 1-4 and 7 See Rule 39.1 (iv) PCT: Methods for treatment of the human or animal body by surgery of therapy as well as diagnostic methods.

2. ☐ Claim numbers..... because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers..... because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

**VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING <sup>2</sup>**

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

**Remark on Protest**

- ☐ The additional search fees were accompanied by applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

US 8702035  
SA 18410

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s)       | Publication<br>date  |
|---|---------------------|----------------------------------|----------------------|
| EP-A- 0140781                             | 08-05-85            | FR-A,B 2553287<br>JP-A- 60115525 | 19-04-85<br>22-06-85 |
| -----                                     |                     |                                  |                      |

**For more details about this annex : see Official Journal of the European Patent Office, No. 12/82**

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